Rapid heparin-sensitive Ca^{2+} release following Ca^{2+}-ATPase inhibition in intact HL-60 granulocytes

Evidence for Ins(1,4,5)P_3-dependent Ca^{2+} cycling across the membrane of Ca^{2+} stores

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In many cell types, emptying of intracellular Ca^{2+} stores after application of inhibitors of the intracellular Ca^{2+}-ATPase (e.g. thapsigargin) is astonishingly rapid. It was the aim of this study to elucidate the underlying mechanism. We first compared thapsigargin-induced emptying of intracellular Ca^{2+} stores in intact and homogenized HL-60 granulocytes. Thapsigargin-induced Ca^{2+} release was rapid in intact cells (33.9±4.9% of store content/min), but it was slow in permeabilized or homogenized cells (7.7±3.9 and 12±3.8% of store content/min respectively). To study whether the Ins(1,4,5)P_3 receptor might be involved in thapsigargin-induced Ca^{2+} release, we tested the effect of heparin, a competitive Ins(1,4,5)P_3 antagonist. In homogenized and permeabilized preparations, heparin did not interfere with thapsigargin-induced Ca^{2+} release. In contrast, when introduced into intact cells by an endocytosis/osmotic-shock procedure, heparin, but not the inactive de-N-sulphated heparin, decreased the rate of Ca^{2+} release by approx. 70%. Heparin inhibited Ca^{2+} release in response to the Ins(1,4,5)P_3-generating receptor agonist *N*-formylmethionyl-leucyl-phenylalanine (f-MLP) (50 nM) and to thapsigargin (50 nM) at comparable concentrations. Heparin inhibition was competitive for f-MLP-induced, but not for thapsigargin-induced, Ca^{2+} release. In permeabilized cells, the addition of low Ins(1,4,5)P_3 concentrations before thapsigargin increased the rate of thapsigargin-induced Ca^{2+} release 4-fold. Taken together, our results suggest that the rapid Ca^{2+}-ATPase-inhibitor-induced Ca^{2+} release is due to a partial activation of the Ins(1,4,5)P_3 receptor in resting cells. This implies Ca^{2+} cycling across the membrane of Ins(1,4,5)P_3-sensitive Ca^{2+} stores in resting cells.

INTRODUCTION

Granulocytes, like virtually all other cell types, contain intracellular Ca^{2+} stores which play an important role in the regulation of the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$). In resting cells, the Ca^{2+}-ATPase of intracellular Ca^{2+} stores sequesters Ca^{2+} from the cytosol and thereby participates in the maintenance of the low resting $[Ca^{2+}]_i$. During cellular activation, Ca^{2+} stores release Ca^{2+} to the cytosol; this Ca^{2+} release is an important mechanism for the stimulated $[Ca^{2+}]_i$ increase (Krause, 1991; Berridge, 1993). Ca^{2+} release from intracellular stores is mediated by two classes of intracellular Ca^{2+}-release channels, Ins(1,4,5)P_3 receptors and ryanodine receptors. In granulocytes, Ca^{2+} release through the Ins(1,4,5)P_3 receptor is the best studied and probably the predominant mechanism (Pittet et al., 1992). It is generally assumed that the Ins(1,4,5)P_3 receptor is inactive in unstimulated cells.

A net release of Ca^{2+} from intracellular stores can be evoked not only by activation of Ca^{2+}-release channels but also by the inhibition of intracellular Ca^{2+}-ATPases (the term 'intracellular Ca^{2+}-ATPase' will be used in this paper to designate the Ca^{2+}-ATPase of intracellular Ca^{2+} stores, as opposed to the Ca^{2+}-ATPase of the plasma membrane). In HL-60 granulocytes (Demaurex et al., 1992), as well as in many other cell types (Kass et al., 1989; Thastrup et al., 1990; Brune and Ullrich, 1991; Mason et al., 1991), three well-defined and structurally unrelated inhibitors of intracellular Ca^{2+}-ATPases, thapsigargin, cyclopiazonic acid and di-t-butylhydroquinone, are able to release Ca^{2+} from intracellular stores. In HL-60 granulocytes, this Ca^{2+} release occurs without an increase in Ins(1,4,5)P_3 levels (Demaurex et al., 1992). The Ca^{2+}-ATPase-inhibitor-induced Ca^{2+} release from intracellular stores is relatively rapid: in HL-60 granulocytes, it starts within 4 s and is completed within some minutes after addition of a Ca^{2+}-ATPase inhibitor (Demaurex et al., 1992). Thus it appears that a permanent Ca^{2+}-ATPase activity compensates for a relatively high permeability of intracellular Ca^{2+} stores in resting cells and that this permeability is revealed by the inhibition of the Ca^{2+}-ATPase.

It was the aim of this study to elucidate the mechanism of the rapid Ca^{2+} release in response to Ca^{2+}-ATPase inhibition, and thus the physiological basis of the high basal permeability of Ca^{2+} stores in intact HL-60 granulocytes.

MATERIALS AND METHODS

Materials

Heparin, de-N-sulphated heparin, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (f-MLP), thapsigargin, MgATP, creatine kinase, phosphocreatine, ionomycin and digitonin were obtained from Sigma (St. Louis, MO, U.S.A.). Di-isopropyl fluorophosphate was from Fluka (Ronkonkoma, NY, U.S.A.), fura-2 from Molecular Probes (Eugene, OR, U.S.A.), Ins(1,4,5)P_3 from L. C. Service Corp. (Woburn, MA, U.S.A.) and $^{45}Ca^{2+}$ from Du Pont de Nemours/NEN (Dreieich, Germany). All other reagents used were of analytical grade. When drugs were added as solutions in dimethyl sulphoxide (DMSO), the final concentration of DMSO in the recording medium did not exceed 0.25%.

For experiments with intact cells two buffers were used. A 'nominally Ca^{2+}-free medium' consisted of NaCl 138 mM, KCl 6 mM, glucose 20 mM, Hepes 20 mM, pH 7.4. The free Ca^{2+} concentration in the nominally Ca^{2+}-free medium was approx. 5 μM, as determined with a Ca^{2+}-sensitive electrode. The medium

Abbreviations used: $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concentration; DMSO, dimethyl sulphoxide; fura-2/AM, fura-2 acetoxymethyl ester; HEDTA, *N*-hydroxyethylethylenediaminetriacetic acid; f-MLP, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine.

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referred to as 'Ca^{2+}-containing medium' contained in addition 1.0 mM $CaCl_2$. The buffers used with homogenates and permeabilized cells are described below.

Culture of HL-60 cells and granulocytic differentiation

HL-60 cells were grown in RPMI 1640 medium supplemented with 10% foetal-calf serum, penicillin (5 units/ml), streptomycin (50 mg/ml) and L-glutamine (2 mM). The cells were replated twice per week. Granulocytic differentiation was obtained by a 7-day incubation with DMSO (Newburger et al., 1979). Final DMSO concentrations were 1.3% (v/v) for day −7 to day −3, and 0.65% for day −2 to the day of the experiment (day 0). The DMSO-differentiated HL-60 cells will be referred to throughout the text as HL-60 granulocytes.

Endocytosis/osmotic-shock procedure

The procedure applied in this study is a slight modification of the original procedure described by Rechsteiner (1992). For this, 40×10^6 HL-60 granulocytes were incubated in 250 μl of a buffer containing 143 mM NaCl, 6 mM KCl, 1 mM $MgSO_4$, 20 mM Hepes, pH 7.4, 5.5 mM glucose, 375 mM sucrose, 7.5% poly-(ethylene glycol) (PEG)-1000 and 7.5% foetal-calf serum. Where indicated, the solutions also contained heparin or de-N-sulphated heparin. The cells were incubated for 15 min at 25 °C to allow fluid-phase endocytosis of extracellular material. To induce hypo-osmotic lysis of endosomes, 4 ml of water was added and cells were incubated under hypo-osmotic conditions for 60 s. Iso-osmolarity was restored by addition of 3.5 ml of 1.8% NaCl. To quantify the efficacy of the endocytosis/osmotic-shock procedure, we introduced Lucifer Yellow (10 mg/ml) or [^{3}H]mannose (10×10^6 c.p.m./ml) into the cytosol by the same method. After three washes, the cytosolic content of Lucifer Yellow or [^{3}H]mannose was measured as the amount of the respective compound that could be released by 20 μM digitonin (Prentki et al., 1984). Assuming a cytosolic volume of 0.5 pl/cell (Demaurex et al., 1993), the cytosolic concentrations were estimated as 2 ± 1 μg/ml for Lucifer Yellow and $(0.1 \pm 0.02) \times 10^6$ c.p.m./ml for [^{3}H]mannose, corresponding to 0.2 ± 0.1% and 1.0 ± 0.2% respectively of the extracellular concentration present during the endocytosis/hypo-osmotic-shock procedure. Inspection of Lucifer Yellow-loaded granulocytes by fluorescence microscopy showed that approx. 90% of the cells had a homogeneous fluorescence, suggesting a predominantly cytosolic localization of the dye. Greater than 90% of the cells excluded Trypan Blue. The method by itself did not interfere with cellular Ca^{2+} homoeostasis. Non-treated and treated cells in a Ca^{2+}-free medium showed respectively (i) a basal $[Ca^{2+}]_i$ of 114.4 ± 5.3 nM and 106.4 ± 11.9 nM, (ii) a peak $[Ca^{2+}]_i$ in response to f-MLP of 258.6 ± 14.3 nM and 273.0 ± 17.1 nM (means ± S.E.M., $n = 3$–9) (for a more detailed description see Jaconi et al., 1993).

Measurement of $[Ca^{2+}]_i$ in intact cells

Details of the procedure have been described previously (Demaurex et al., 1994). HL-60 granulocytes were loaded with 2 μM fura-2/AM (acetoxymethyl ester) (37 °C for 45 min). Experiments were performed on a Perkin–Elmer fluorimeter (LS3; Perkin–Elmer, Cetus), thermostatically maintained at 37 °C. Fluorescence emission was set at 505 nm, and fluorescence excitation at 340 nm. Ca^{2+} release in response to ionomycin, thapsigargin or f-MLP was measured as the peak $[Ca^{2+}]_i$ value after addition of the respective compound in a nominally Ca^{2+}-free buffer. In preliminary experiments, we have compared this method with the determination of the area under the curve and have observed identical results. Ca^{2+} influx in response to thapsigargin and f-MLP was measured as the $[Ca^{2+}]_i$ increase observed during the first 10 s after Ca^{2+} re-addition to cells stimulated for 5 min in a nominally Ca^{2+}-free medium. To quantify the time course of emptying of intracellular Ca^{2+} stores by thapsigargin, we have added ionomycin at the indicated time after stimulation of cells with thapsigargin in a nominally Ca^{2+}-free medium. We defined 100% filling of Ca^{2+} stores as the amount of Ca^{2+} released by ionomycin at zero time (i.e. ionomycin was added instead of thapsigargin). The initial rate of Ca^{2+} release was calculated as the percentage of Ca^{2+}-store content released during the first 1 min of thapsigargin addition.

Preparation of cell homogenates

DMSO-differentiated HL-60 cells were harvested and treated with 5 μM of the protease inhibitor di-isopropyl fluorophosphate, washed, re-suspended in KCl/Hepes buffer (KCl 130 mM, Hepes 20 mM, pH 7.4), 1 mM MgATP, a cocktail of protease inhibitors, (aprotinin 80 nM, pepstatin A 0.7 mM, leupeptin 1 mM, phenylmethanesulphonyl fluoride 0.25 mM, benzamidine 0.8 mM) and the antioxidant dithiothreitol 1 mM. Cells were then disrupted by nitrogen cavitation. Nuclei and non-disrupted cells were removed by centrifugation at 80 *g* for 10 min. Protein was determined by a modification of the Lowry procedure (Peterson, 1977). For more detailed description of the method see Van Delden et al. (1992).

Permeabilization of HL-60 granulocytes

To permeabilize the plasma membrane of HL-60 granulocytes, we added 40 μM digitonin 5 min before initiation of Ca^{2+} uptake. Under these conditions, more than 90% of cells were permeabilized (as assessed by uptake of Trypan Blue). However, in a Ca^{2+}-free medium, no β-glucuronidase release was observed (results not shown), suggesting a selective permeabilization of the plasma membrane.

$^{45}Ca^{2+}$ technique

Ca^{2+}-flux measurements were conducted in both permeabilized cells and homogenates by the $^{45}Ca^{2+}$ technique. Intact HL-60 granulocytes (2×10^6 cells/ml) or homogenates (250 μg/ml) were preincubated for 10 min at 30 °C in a buffer mimicking intracellular ionic conditions (KCl 120 mM, $MgCl_2$ 1 mM, Hepes 25 mM, pH 7.0) in the presence of an ATP-regenerating system (MgATP 1 mM, phosphocreatine 2.5 mM, creatine kinase 4 units/ml) and mitochondrial inhibitors (antimycin 0.2 μM and oligomycin 1 mg/ml). The buffer contained 1 mM *N*-hydroxyethylethylenediaminetriacetic acid (HEDTA), and Ca^{2+} uptake was initiated by addition of 200 nCi/ml $^{45}Ca^{2+}$ and 8 μM unlabelled Ca^{2+} (free $[Ca^{2+}] = 80$ nM) or 20 μM unlabelled Ca^{2+} (free $[Ca^{2+}] = 200$ nM). If not otherwise stated, $^{45}Ca^{2+}$ experiments were performed in 80 nM free Ca^{2+}. The times allowed for Ca^{2+} uptake and the addition of various active compounds [thapsigargin, Ins(1,4,5)P_3, heparin] are indicated in the Figure legends. Zero time in the Figures usually denotes the time of thapsigargin addition. At the indicated time, 100 μl samples were taken in duplicate, transferred on to a 0.45 μm filter (Millipore, HA type) and washed with 3×5 ml of a buffer containing 120 mM KCl, 1 mM $LaCl_3$ and 20 mM Hepes, pH 7.0. Filters were placed in a vial containing a liquid-scintillation mixture (Ultima Gold, Packard) and the radioactivity was measured in a Packard 1900 TR scintillation counter.

RESULTS

Thapsigargin-induced Ca^{2+} release in intact and homogenized HL-60 granulocytes

To understand the mechanism of the net release of Ca^{2+} following Ca^{2+}-ATPase inhibition, we first compared thapsigargin-induced Ca^{2+} release in intact and homogenized HL-60 granulocytes. In the present studies the most potent Ca^{2+}-ATPase inhibitor, thapsigargin, was used, but similar results (not shown) were obtained with cyclopiazonic acid or di-t-butylhydroquinone. We used $^{45}Ca^{2+}$ fluxes to study Ca^{2+} regulation in homogenized cells, and fura-2 to study Ca^{2+} regulation in intact cells. The two different techniques appear to be the most appropriate approaches to study Ca^{2+} regulation in these two different preparations. However, the use of two distinct techniques raises questions with respect to the quantitative comparison of the results. Indeed, $^{45}Ca^{2+}$-flux studies in permeabilized cells measure unidirectional fluxes, whereas the fura-2 measurements in intact cells represent net fluxes. We have therefore designed experiment protocols that measure the amount of ionomycin-releasable Ca^{2+} after a given time of exposure to thapsigargin. Even in intact cells, this protocol mostly detects the unidirectional fluxes from the stores to the cytosol, because of (i) the rapidity of the ionomycin-induced Ca^{2+} release and (ii) the inhibition of the intracellular Ca^{2+}-ATPase by thapsigargin. Experiments were performed in a nominally Ca^{2+}-free medium (intact cells), or at 80 nM free Ca^{2+} (homogenates). The free Ca^{2+} concentration in the nominally Ca^{2+}-free medium was approx. 5 μM. This condition was chosen because it precluded stimulated Ca^{2+} influx but did not lead to spontaneous depletion of Ca^{2+} stores (Demaurex et al., 1994). The Ca^{2+} concentration of 80 nM in the experiments with homogenates was chosen because it is close to the basal Ca^{2+} concentration of HL-60 cells in a nominally Ca^{2+}-free medium (see below). Zero time was defined as the time of thapsigargin addition. The Ca^{2+} content of intracellular Ca^{2+} stores was defined as the ionomycin-releasable Ca^{2+} (peak of ionomycin-induced $[Ca^{2+}]_i$ increase for intact cells, the amount of ionomycin-releasable $^{45}Ca^{2+}$ for homogenates) and is shown as percentage of the initial content (content at zero time). As shown in Figure 1 (white squares), the addition of thapsigargin to intact cells led to a rapid depletion of intracellular Ca^{2+} stores; more than 80% of the total content of intracellular Ca^{2+} stores was released within 4 min. In contrast, thapsigargin induced only a slow Ca^{2+} release in homogenized cells. The initial rate of thapsigargin-induced Ca^{2+} release in intact cells was 33.9 ± 4.9% of the store content/min. In contrast, a slow, long-lasting, release (initial rate 12.0 ± 3.8% of store content/min) was observed in homogenates (Figure 1, white circles).

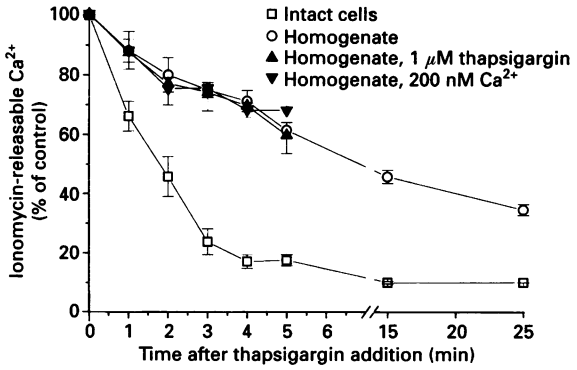

Figure 1 Kinetics of thapsigargin-induced depletion of intracellular Ca^{2+} stores in intact and homogenized HL-60 granulocytes

Measurements were performed by using the fluorescent Ca^{2+} indicator fura-2 in intact HL-60 granulocytes and the $^{45}Ca^{2+}$ technique in homogenates (for detailed description see the Materials and methods section). In both preparations the Ca^{2+} content of intracellular Ca^{2+} stores was defined as ionomycin-releasable Ca^{2+} (i.e. for intact cells, the peak $[Ca^{2+}]_i$ increase upon ionomycin addition in a nominally Ca^{2+}-free medium, and for homogenates the amount of ionomycin-releasable $^{45}Ca^{2+}$). $^{45}Ca^{2+}$ experiments were performed in a buffer with a free $[Ca^{2+}]$ of 80 nM, or, if indicated, 200 nM. Thapsigargin was added to cells (50 nM) or homogenates (100 nM, or, if indicated, 1 μM) at zero time. Ionomycin-releasable Ca^{2+} (i.e. Ca^{2+} content of Ca^{2+} stores) was expressed as a function of time after thapsigargin addition; 100% corresponds to the values at the time of thapsigargin addition. The results are means $\pm$ S.E.M. of three experiments (the 100% value of ionomycin-induced $[Ca^{2+}]_i$ increase in intact cells was 970 ± 230 nM; the 100% value of the amount of ionomycin-releasable Ca^{2+} in homogenates was 1.74 ± 0.12 nmol of Ca^{2+}/mg of protein).

We have previously shown that maximal thapsigargin-induced Ca^{2+} release in intact cells is observed at thapsigargin concentrations around 30–50 nM (Demaurex et al., 1992). As thapsigargin is a hydrophobic compound, and the phospholipid concentration during the experiments with homogenates was higher than during the experiments with intact cells, the relatively slow Ca^{2+} release in homogenates might be due to sub-maximal thapsigargin concentrations. However, (i) 10-fold higher thapsigargin concentration did not increase the rate of Ca^{2+} release in intact cells (Figure 1, upright triangles) and (ii) addition of 0.1 μM thapsigargin before initiation of Ca^{2+} uptake completely blocked Ca^{2+} accumulation (results not shown). The thapsigargin-induced Ca^{2+} release might depend on $[Ca^{2+}]_i$, which increases during the experiments with intact cells, but was clamped by a Ca^{2+} buffer during the experiments with homogenized cells. However, a similarly slow Ca^{2+} release was observed when the experiments with homogenates were performed in 200 nM Ca^{2+} (Figure 1, inverted triangles). Thus, it appears that even supra-maximal thapsigargin concentrations do not induce a rapid Ca^{2+} release by themselves, and that in intact cells there is a substantially increased thapsigargin-induced Ca^{2+} release, which may not be explained by an increase in $[Ca^{2+}]_i$.

Effect of heparin on thapsigargin-induced Ca^{2+} release

The results obtained so far suggest that the permeability of intracellular Ca^{2+} stores is higher in intact than in homogenized cells. In HL-60 granulocytes, the Ins(1,4,5)P_3 receptor is probably the predominant pathway of Ca^{2+} permeation from the lumen to the outside of Ca^{2+} stores. We therefore wondered whether the thapsigargin-induced Ca^{2+} release in intact HL-60 granulocytes might be explained by a partial activation of the Ins(1,4,5)P_3 receptor in unstimulated HL-60 granulocytes. Indeed, previous studies from our laboratory have found relatively high Ins(1,4,5)P_3 concentrations (280 nM) in unstimulated HL-60 granulocytes (Pittet et al., 1989). The most widely used compound to block the action of Ins(1,4,5)P_3 on its receptor is heparin. Heparin is a competitive and reversible blocker of Ins(1,4,5)P_3 binding to its receptor and of Ins(1,4,5)P_3-induced Ca^{2+} release (Ghosh et al., 1988). As expected, heparin blocked Ins(1,4,5)P_3-induced Ca^{2+} release, but not thapsigargin-induced Ca^{2+} release in homogenates of HL-60 granulocytes (Figures 2a and 2b). To investigate the effect of heparin in intact cells, we introduced heparin into the cytosol of HL-60 granulocytes, using an endocytosis/osmotic-shock procedure. This technique allows the introduction of macromolecules into the cytoplasm of cells without disruption of the plasma membrane (Rechsteiner, 1992). In granulocytes, this method does not interfere with cellular Ca^{2+} homoeostasis (Jaconi et al., 1993) or complex motile functions (Hendey et al., 1992), indicating that cellular integrity is preserved. The cytosolic concentrations of the compound of interest

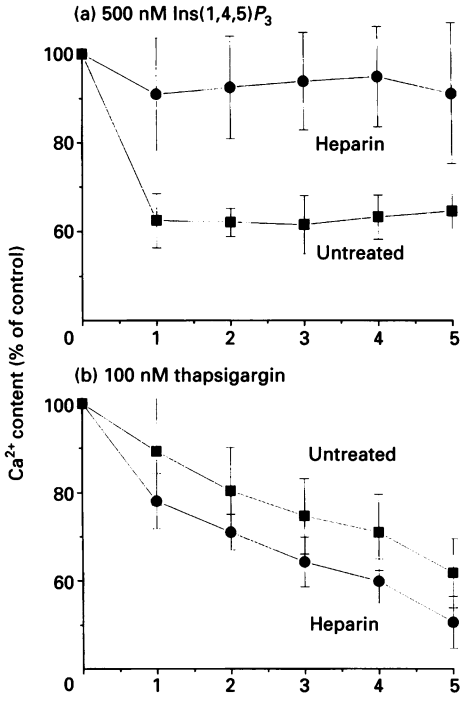

Figure 2 Effect of heparin on Ins(1,4,5)P_3- and thapsigargin-induced Ca^{2+} release from HL-60 homogenates

Thapsigargin- and Ins(1,4,5)P_3-induced Ca^{2+} release from HL-60 homogenates was studied by the $^{45}Ca^{2+}$ technique; 100% was defined as the Ca^{2+} content of stores at zero time, which was the time after 13 min of ATP-dependent Ca^{2+} accumulation. Heparin (200 μg/ml) (circles) or solvent (squares) was added at time −3 min; 500 nM Ins(1,4,5)P_3 (**a**) or 100 nM thapsigargin (**b**), was added at zero time. The 100% value corresponds to 1.36 ± 0.04 nmol of Ca^{2+}/mg of protein. Results are means ± S.E.M. of three independent experiments performed in duplicate.

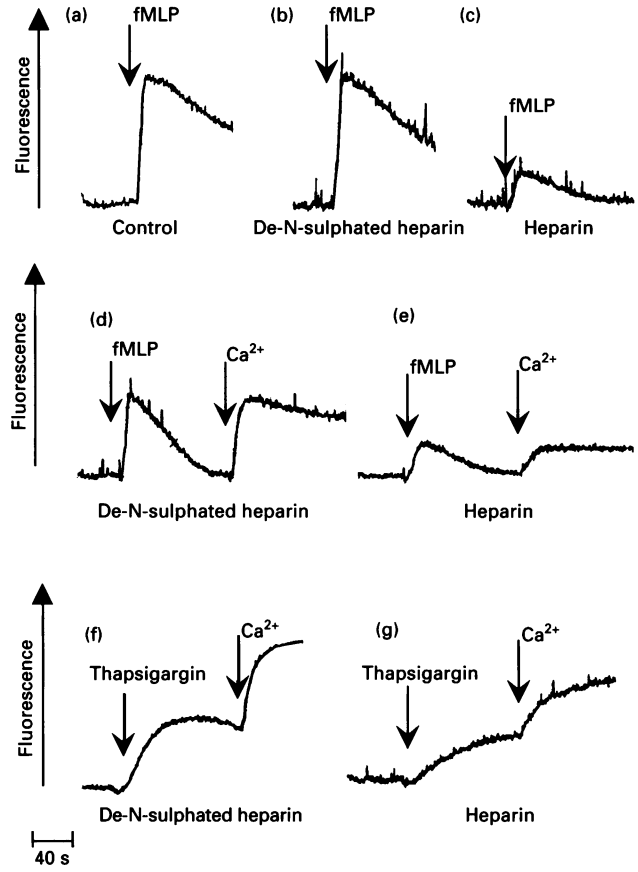

Figure 3 Effect of cytosolic heparin on f-MLP- and thapsigargin-induced $[Ca^{2+}]_i$ elevations in intact cells

Heparin (**c**, **e**, **g**) or de-N-sulphated heparin (**b**, **d**, **f**) was introduced into intact HL-60 granulocytes by using the endocytosis/osmotic-shock procedure. The concentration of heparin and de-N-sulphated heparin during the procedure was 10 mg/ml, yielding intracellular concentrations of approx. 20–100 μg/ml (see the Materials and methods section). Control cells were exposed to the same procedure in the absence of heparin (**a**). Cells were then loaded with the fluorescent Ca^{2+} indicator fura-2, and $[Ca^{2+}]_i$ was measured fluorimetrically. (**a**, **b**, **c**) Cells were stimulated with 50 nM f-MLP in a Ca^{2+}-containing medium; (**d**, **e**) cells were stimulated with 50 nM f-MLP in a nominally Ca^{2+}-free medium; (**f**, **g**) cells were stimulated with 50 nM thapsigargin in a nominally Ca^{2+}-free medium. In traces (**d**) and (**e**), 2 mM $CaCl_2$ (indicated by the arrow 'Ca^{2+}') was added to the extracellular solution 5 min after stimulation with the respective agonist to assess stimulated Ca^{2+} influx. The traces were recorded on a strip-chart recorder and are therefore shown as relative fluorescence units. For time points of interest, the absolute $[Ca^{2+}]_i$ values were calculated and are given in the legend of Figure 5 (stimulated values) or in the text (basal values). Traces shown are typical for at least 3 independent experiments.

obtained with this method were between 0.2 and 1% of the concentrations present in the extracellular solution during the procedure (see the Materials and methods section for details). As heparin is a polyanion, it may cause non-specific charge effects. We have therefore used in several control experiments a de-N-sulphated heparin, which has a comparable amount of negative charges, but is inactive on the Ins(1,4,5)P_3 receptor (Ghosh et al., 1988). We first tested the effect of heparin loading on Ca^{2+} signalling induced by the receptor agonist f-MLP, which acts through phospholipase C activation and Ins(1,4,5)P_3 generation. For the experiments shown in Figure 3, we used 10 mg/ml heparin in the loading buffer, yielding predicted cytosolic heparin concentrations of between 20 and 100 μg/ml. The f-MLP-induced $[Ca^{2+}]_i$ elevations were inhibited by heparin, whether assessed in a Ca^{2+}-containing medium (Figures 3a and 3c) or in a Ca^{2+}-free medium (Figures 3d and 3e). Similarly, the f-MLP-stimulated Ca^{2+} influx (assessed as Ca^{2+} re-addition after stimulation with f-MLP in a Ca^{2+}-free medium) was markedly diminished in heparin-loaded cells (Figures 3d and 3e, arrow 'Ca^{2+}'). The de-N-sulphated heparin did not interfere with f-MLP-induced Ca^{2+} signalling (Figures 3b and 3d), indicating that the heparin effect was specific. Having established the efficacy of the heparin loading on a well-known Ins(1,4,5)P_3-dependent signalling pathway, we next investigated the heparin effect on the thapsigargin-induced Ca^{2+} signalling. As opposed to the observations in homogenates (Figure 2b), heparin clearly inhibited thapsigargin-induced Ca^{2+} release in intact cells (Figures 3f and 3g). Both the f-MLP- and thapsigargin-stimulated Ca^{2+} influx were also inhibited by heparin (Figures 3f and 3g, arrow 'Ca^{2+}'). The heparin concentrations in the loading buffer necessary for half-maximal inhibition of f-MLP-induced Ca^{2+} release and Ca^{2+} influx, and thapsigargin-induced Ca^{2+} release and Ca^{2+} influx, were very similar, being 3.6 ± 1, 2.3 ± 0.4, 3.1 ± 0.7 and 2.7 ± 1.3 mg/ml heparin in the loading buffer, respectively (n = 3–4, means ± S.E.M.) (Figure 4). The heparin inhibition of f-MLP- and thapsigargin-induced Ca^{2+} release is best explained by a direct effect on the Ins(1,4,5)P_3-sensitive Ca^{2+} channel. The inhibition of Ca^{2+} influx is most likely secondary to the inhibition of Ca^{2+} release: in granulocytes the emptying of intracellular Ca^{2+} stores is the predominant, if not the only, signal for Ca^{2+} influx (Demaurex et al., 1994). Note that heparin did not interfere with the amount of ionomycin-releasable Ca^{2+} (Figure 4a,

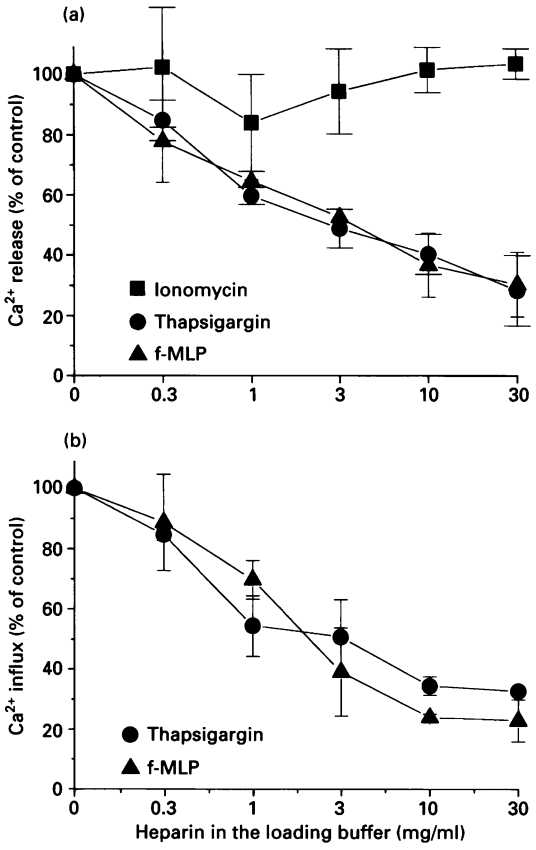

Figure 4 Dose-dependence of the heparin inhibition of f-MLP- and thapsigargin-induced Ca^{2+} signalling in intact cells

Heparin was introduced into intact cells by the endocytosis/osmotic-shock procedure. The concentrations shown on the abscissa correspond to the heparin concentrations in the loading buffer; the intracellular concentrations obtained were estimated to be approx. 0.2–1% of the concentrations in the loading buffer (see the Materials and methods section). Cells were loaded with fura-2 and fluorimetric $[Ca^{2+}]_i$ measurements were performed. (**a**) Ca^{2+} release (peak of $[Ca^{2+}]_i$ increase in a nominally Ca^{2+}-free medium) in response to 1 μM ionomycin, 50 nM thapsigargin, or 50 nM f-MLP (100% corresponds to $[Ca^{2+}]_i$ increases of 503.5 ± 22.5, 86.1 ± 13.3 and 122.7 ± 16.1 nM for ionomycin, thapsigargin and f-MLP respectively). (**b**) Ca^{2+} influx (rate of $[Ca^{2+}]_i$ increase after Ca^{2+} re-addition 5 min after stimulation in a Ca^{2+}-free medium) in response to 50 nM thapsigargin or 50 nM f-MLP (100% values were 206.1 ± 27.7 and 125.3 ± 35.1 nM for thapsigargin and f-MLP respectively). Values are means $\pm$ S.E.M. of three different experiments.

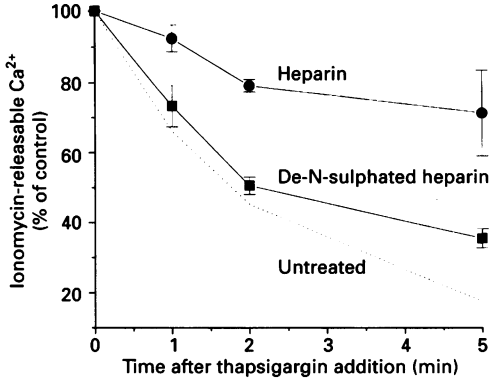

Figure 5 Effect of heparin on the kinetics of thapsigargin-induced store depletion in intact HL-60 cells

Heparin or de-N-sulphated heparin was introduced into intact cells by the endocytosis/osmotic-shock procedure; the heparin concentrations in the loading buffer were 3 mg/ml and are estimated to yield intracellular concentrations between 6 and 30 μg/ml (see the Materials and methods section). Cells were loaded with fura-2, and fluorimetric $[Ca^{2+}]_i$ measurements were performed. The thapsigargin-induced decrease in Ca^{2+} content of intracellular Ca^{2+} stores (ionomycin-releasable Ca^{2+}) in HL-60 granulocytes loaded with heparin (circles) or de-N-sulphated heparin (squares) is shown. The broken line shows, for comparison, results for HL-60 granulocytes that were not subjected to the endocytosis/osmotic-shock procedure. The results are means $\pm$ S.E.M. of three separate experiments (100% corresponds to a $[Ca^{2+}]_i$ increase of 970 ± 230 nM in response to ionomycin).

Table 1 Initial rate of thapsigargin-induced emptying of intracellular Ca^{2+} stores under various experimental conditions

Data shown in this Table are derived from the experiments described in Figure 1 (intact cells, control), Figure 5 (intact cells, heparin and de-N-sulphated heparin) and Figure 7 (permeabilized cells). The decrease in the amount of ionomycin-releasable Ca^{2+} in the first 1 min after thapsigargin addition was measured to calculate the initial rate of thapsigargin-induced emptying of intracellular Ca^{2+} stores (for details of the experiments see legends to Figures 1, 5 and 7). The thapsigargin concentrations used were 50 nM and 100 nM in intact and permeabilized cells respectively. Heparin and de-N-sulphated heparin were introduced into intact cells by the endocytosis/osmotic-shock procedure. Concentrations in the loading buffer were 3 mg/ml, yielding estimated cytosolic concentrations between 6 and 30 μg/ml. In permeabilized cells, Ins(1,4,5)P_3 (100 nM) was added 3 min before thapsigargin addition, and heparin (200 μg/ml) 6 min before thapsigargin addition. Data are means $\pm$ S.E.M. of 3 independent experiments (intact cells) or 3 independent experiments performed in duplicate (permeabilized cells).

	Intact cells (% of content/min)		Permeabilized cells (% of content/min)
Control	33.9 ± 4.9	Control	7.7 ± 3.9
Heparin	7.7 ± 3.7	Ins(1,4,5)P_3	33.5 ± 4.7
De-N-sulphated heparin	26.8 ± 5.9	Heparin + Ins(1,4,5)P_3	5.9 ± 3.7

squares), excluding the possibility that the diminished Ca^{2+} release in response to f-MLP and thapsigargin is due to a depletion of Ca^{2+} stores by heparin. It should, however, be mentioned that heparin slightly, but consistently, increased the basal $[Ca^{2+}]_i$ in HL-60 granulocytes. Basal $[Ca^{2+}]_i$ concentrations were 105.2 ± 4.1, 105.2 ± 4.0, 116.4 ± 4.0, 126.2 ± 8.2, 130 ± 4.0, 139.5 ± 5.5 and 140.2 ± 7.1 nM, when cells were loaded with 0, 0.1, 0.3, 1, 3, 10 and 30 mg/ml heparin in the loading buffer, respectively (means $\pm$ S.E.M. from 8 determinations in 3 independent experiments). This increase in basal $[Ca^{2+}]_i$ was not due to an increased plasma-membrane Ca^{2+} permeability, because the experiments were done in the absence of extracellular Ca^{2+}. As shown in Figure 4(a) (squares), it was also not due to a Ca^{2+} release from intracellular Ca^{2+} stores. Thus the small increase in basal $[Ca^{2+}]_i$ is most likely due to a moderate inhibition of a Ca^{2+}-extrusion mechanism of the plasma membrane (e.g. the plasma-membrane Ca^{2+}-ATPase).

To quantify the effect of heparin on thapsigargin-induced Ca^{2+} release, we have studied the effect of thapsigargin on the content of Ca^{2+} stores, using the experimental protocol described in Figure 1. As shown in Figure 5, cells loaded with de-N-sulphated heparin showed a rapid emptying of Ca^{2+} stores and an initial Ca^{2+} release, with a rate of 26.8 ± 5.9% of store content/min, similar to results seen in untreated control cells. In contrast, cells loaded with heparin showed a slow emptying of Ca^{2+} stores and an initial Ca^{2+} release with a rate of approx. 7.7 ± 5.9% of store content/min (Table 1).

As f-MLP increases Ins(1,4,5)P_3 in a dose-dependent fashion (Pittet et al., 1990) and heparin is a competitive blocker of Ins(1,4,5)P_3 binding to its receptor (Ghosh et al., 1988), one

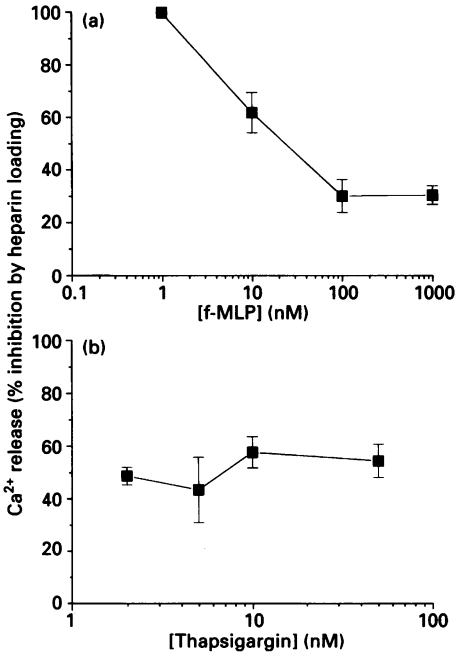

Figure 6 Competitive inhibition of f-MLP-induced, but not of thapsigargin-induced, Ca^{2+} release by heparin

Heparin was introduced into intact cells by the endocytosis/osmotic-shock procedure; the heparin concentrations in the loading buffer were 3 mg/ml and are estimated to yield intracellular concentrations between 6 and 30 μg/ml (see the Materials and methods section). Control cells were exposed to the same procedure in the absence of heparin. Cells were loaded with fura-2, and Ca^{2+} release ($[Ca^{2+}]_i$ increase in a nominally Ca^{2+}-free medium) in response to different f-MLP concentrations (**a**) or thapsigargin concentrations (**b**) was determined fluorimetrically in cells. In control cells, $[Ca^{2+}]_i$ increases in response to 1, 10, 100, and 1000 nM f-MLP were 30 ± 3, 153 ± 25, 178 ± 50 and 349 ± 54 nM respectively, and $[Ca^{2+}]_i$ increases in response to 2, 5, 10 and 50 nM thapsigargin were 30 ± 7, 65 ± 21, 139 ± 18 and 203 ± 14 nM respectively. The data are expressed as percentage inhibition of f-MLP-induced (**a**) and thapsigargin-induced (**b**) Ca^{2+} release in heparin-loaded as compared with control cells. Results are means $\pm$ S.E.M. of three independent experiments.

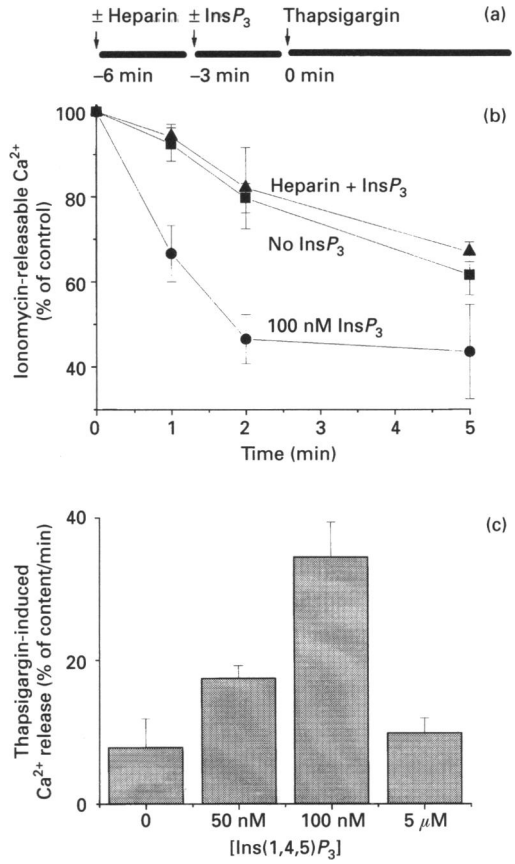

Figure 7 Effect of preincubation with low Ins(1,4,5)P_3 concentrations on the kinetics of thapsigargin-induced Ca^{2+} release in permeabilized HL-60 granulocytes

Thapsigargin-induced Ca^{2+} release from permeabilized HL-60 granulocytes was studied by the $^{45}Ca^{2+}$ technique. Cells were permeabilized by addition of 40 μM digitonin before initiation of Ca^{2+} uptake. Zero time was defined as the time after 13 min of ATP-dependent Ca^{2+} accumulation. (**a**) Experimental protocol: heparin was added at -6 min, Ins(1,4,5)P_3 at -3 min, and thapsigargin at 0 min. (**b**) Heparin (200 μg/ml) (triangles) or solvent (squares and circles) was added at -6 min, 100 nM Ins(1,4,5)P_3 (circles and triangles) or solvent (squares) at -3 min, and 100 nM thapsigargin at 0 min. Ionomycin-releasable Ca^{2+} (i.e. Ca^{2+} content of Ca^{2+} stores) was expressed as a function of time after thapsigargin addition; 100% corresponds to the values at the time of thapsigargin addition (0.52 ± 0.02, 0.28 ± 0.03 and 0.53 ± 0.08 nmol of $Ca^{2+}/10^7$ cells, for the conditions 'No InsP_3' 'InsP_3' and 'Heparin + InsP_3' respectively). (**c**) Initial rate of thapsigargin-induced Ca^{2+} release after incubation with 0, 50 nM, 100 nM and 5 μM Ins(1,4,5)P_3. The average store content before Ins(1,4,5)P_3 addition was 0.47 ± 0.06 nmol of $Ca^{2+}/10^7$ cells; 50 nM, 100 nM and 5 μM Ins(1,4,5)P_3 released 25.5 ± 8.8, 51.5 ± 7.3 and 69.7 ± 3.5% of total store content respectively. The results are means $\pm$ S.E.M. of three to four independent experiments performed in duplicate.

would expect that the efficacy of the heparin inhibition would decrease with higher f-MLP concentrations. In contrast, as thapsigargin does not generate Ins(1,4,5)P_3 (Demaurex et al., 1992), but potentially acts through constant basal Ins(1,4,5)P_3 levels, one would expect that the efficacy of the heparin block is independent of the thapsigargin concentration. As shown in Figure 6, heparin acted indeed as a competitive inhibitor of f-MLP-induced $[Ca^{2+}]_i$ signalling, but not of thapsigargin-induced Ca^{2+} signalling. This further strengthens the evidence that the heparin effects observed in this study are indeed due to a block of the Ins(1,4,5)P_3 effect on its receptor.

Effect of low Ins(1,4,5)P_3 concentrations on thapsigargin-induced Ca^{2+} release in permeabilized cells

If the rapid thapsigargin-induced Ca^{2+} release in intact cells is indeed due to the basal Ins(1,4,5)P_3 concentrations, we should be able to increase the rate of thapsigargin-induced Ca^{2+} release in homogenized cell preparations by addition of low Ins(1,4,5)P_3 concentrations. As the sensitivity of the thapsigargin-induced Ca^{2+} release might critically depend on the structural integrity of intracellular Ca^{2+} stores, we used digitonin-permeabilized cells, rather than cell homogenates, for these studies. As shown in Figure 7, thapsigargin-induced Ca^{2+} release in permeabilized cells resembles thapsigargin-induced Ca^{2+} release in homogenates with an initial rate of release of 7.7 ± 3.9% of store content/min. This would be compatible with the concept that the high permeability of intracellular Ca^{2+} stores is due to a soluble messenger that is able to diffuse through the digitonin-induced pores in the plasma membrane. As the ratio of the intracellular volume to the extracellular volume is at least 1:100 under our assay conditions, such a soluble messenger would be sufficiently diluted to be inactive. We therefore added relatively low Ins(1,4,5)P_3 concentrations to permeabilized HL-60 granulocytes and tested their effect on thapsigargin-induced Ca^{2+} release. When 50 or 100 nM Ins(1,4,5)P_3 was added 3 min before thapsigargin, the initial rate of thapsigargin-induced Ca^{2+} release increased to, respectively, 17.1 ± 1.7 and 33.5 ± 4.7% of store content/min (Figure 7 and Table 1). Thus, in permeabilized cells,

relatively low Ins(1,4,5)P_3 concentrations sensitize the intracellular Ca^{2+} stores to Ca^{2+} release by thapsigargin. The Ins(1,4,5)P_3 effect could be completely prevented by the addition of heparin (200 μg/ml; Figure 7 and Table 1). Importantly, the Ins(1,4,5)P_3 effect was entirely lost when Ins(1,4,5)P_3-sensitive Ca^{2+} stores were entirely depleted by 5 μM Ins(1,4,5)P_3. This demonstrates that the Ins(1,4,5)P_3 enhancement of thapsigargin-induced Ca^{2+} release is due to Ca^{2+} cycling across the membrane of Ins(1,4,5)P_3-sensitive Ca^{2+} stores, and not to a high permeability of Ins(1,4,5)P_3-insensitive Ca^{2+} stores.

DISCUSSION

Our results demonstrate that: (i) the rate of Ca^{2+}-ATPase-inhibitor-induced Ca^{2+} release is substantially different in homogenized and intact cells; (ii) intracellular heparin is able to block Ca^{2+}-ATPase-inhibitor-induced Ca^{2+} release in intact, but not in homogenized, cells; and (iii) low Ins(1,4,5)P_3 concentrations increase the rate of Ca^{2+}-ATPase-inhibitor-induced Ca^{2+} release in permeabilized cells, yielding rates comparable with those for intact cells. Taken together, these results strongly suggest that the rapid Ca^{2+}-ATPase-inhibitor-induced Ca^{2+} release observed in unstimulated HL-60 granulocytes is due to a partial activation of the Ins(1,4,5)P_3-sensitive Ca^{2+}-release channel.

The difference in the rate of thapsigargin-induced Ca^{2+} release in intact cells and homogenates is striking, and has been observed in other cellular systems (Missiaen et al., 1993). The fact that the slow thapsigargin-induced Ca^{2+} release was observed not only in homogenates, but also after digitonin permeabilization of HL-60 granulocytes, suggests that it is not due to disruption of the continuity of Ca^{2+} stores by the homogenization procedure. It rather hints at the importance of a soluble factor, potentially Ins(1,4,5)P_3, for maintaining the high permeability of Ca^{2+} stores in intact cells. To investigate a possible role of the Ins(1,4,5)P_3 receptor in the Ca^{2+}-ATPase-inhibitor-induced Ca^{2+} release, we have introduced heparin into HL-60 granulocytes, using an endocytosis/hypo-osmotic-shock procedure. Heparin potently competes with Ins(1,4,5)P_3 for binding to the Ins(1,4,5)P_3-sensitive Ca^{2+} channel and is at present the most widely used Ins(1,4,5)P_3 antagonist. Compatible with a role of the Ins(1,4,5)P_3 receptor, heparin indeed blocked Ca^{2+}-ATPase inhibitor-induced Ca^{2+} release. However, although heparin is a potent blocker of the Ins(1,4,5)P_3 receptor, it is not specific (see, e.g., Smith et al., 1992).

The following observations, however, suggest that the observed inhibition of thapsigargin-induced Ca^{2+} release by heparin may indeed be attributed to its effect on the Ins(1,4,5)P_3 receptor: (i) heparin did not block thapsigargin-induced Ca^{2+} release in homogenates or permeabilized cells; (ii) the dose–inhibition curves for heparin were comparable for Ca^{2+} release in response to the Ins(1,4,5)P_3-generating receptor-agonist f-MLP and for thapsigargin-induced Ca^{2+} release; (iii) as predicted, heparin acted as a competitive antagonist for f-MLP-induced Ca^{2+} release, but as a non-competitive antagonist for thapsigargin-induced Ca^{2+} release; (iv) a rapid heparin-sensitive thapsigargin-induced Ca^{2+} release could be reconstituted in permeabilized cells, by addition of low Ins(1,4,5)P_3 concentrations (Figure 7; see also Missiaen et al., 1993; Loomis-Husselbee and Dawson, 1993). In addition, previous studies have demonstrated relatively high basal Ins(1,4,5)P_3 concentrations (280 ± 110 nM) in unstimulated HL-60 granulocytes (Pittet et al., 1989).

In this study, we have analysed the effect of thapsigargin in intact cells and homogenates, the effect of heparin on f-MLP-induced and thapsigargin-induced Ca^{2+} release in intact cells, as well as the effect of Ins(1,4,5)P_3 levels on thapsigargin-induced Ca^{2+} release in permeabilized cells. The different experimental approaches consistently show that (i) thapsigargin-induced Ca^{2+} release is markedly enhanced by low Ins(1,4,5)P_3 concentrations, and (ii) heparin blocks thapsigargin-induced Ca^{2+} release through blocking the Ins(1,4,5)P_3 action, and not through a different mechanism. However, although this qualitative comparison is convincingly supported by our data, caution is necessary with respect to the quantitative comparison of the results obtained with the different systems: (i) whereas $^{45}Ca^{2+}$ fluxes represent unidirectional fluxes, the fura measurements in intact cells represent net fluxes (even if our experimental protocol was designed in a way that minimizes these differences; see the Results section); (ii) the measurement of f-MLP-induced Ca^{2+} release includes a large component of reuptake by the intracellular Ca^{2+}-ATPase, whereas this is not the case for thapsigargin-induced Ca^{2+} release; and (iii) the affinity of Ins(1,4,5)P_3-induced Ca^{2+} release is most likely not identical in permeabilized cells and in intact cells [the basal Ins(1,4,5)P_3 concentration measured in intact HL-60 cells, i.e. 250 nM (Pittet et al., 1989), would already empty most of the Ins(1,4,5)P_3-sensitive Ca^{2+} pool when added to permeabilized cells].

From a biological point of view, the most important result of our study is the evidence for Ca^{2+} cycling across the membrane of Ca^{2+} stores. The concept of Ca^{2+} cycling implies the entry of Ca^{2+} into the cytosol through a channel and the rapid extrusion by a Ca^{2+} pump, with the consequent generation of a localized $[Ca^{2+}]$ increase in proximity of the channel, but no generalized increase in $[Ca^{2+}]_i$. Ca^{2+} cycling was first proposed to occur across the plasma membrane and to play a role in the sustained cellular response to hormonal stimulation (Rasmussen et al., 1989). Our study in HL-60 granulocytes suggests that Ca^{2+} cycling may also occur at the membrane of intracellular Ca^{2+} stores, even without previous cellular stimulation. This Ca^{2+} cycling implies a permanent use of cellular metabolic energy, raising the question of its possible physiological importance. At least two possible physiological roles of Ca^{2+} cycling are apparent: (i) localized $[Ca^{2+}]_i$ increases and (ii) sensitivity of $[Ca^{2+}]_i$ to modulation of Ca^{2+}-ATPase activity. Ca^{2+} cycling across the Ca^{2+}-store membrane would be expected to generate a localized $[Ca^{2+}]_i$ increase in close proximity to the Ins(1,4,5)P_3 receptor. Thus Ca^{2+}-sensitive elements that are located in close proximity to the Ins(1,4,5)P_3 receptor might be regulated by Ca^{2+} cycling. Ca^{2+} cycling across the membrane of Ca^{2+} stores might, for example, be important for protein synthesis, cellular proliferation and other more permanent cellular functions that are not directly linked to receptor activation. It might also be important for the regulation of the intra-mitochondrial Ca^{2+} concentration, which has been recently shown to be sensitive to micro-domains of high Ca^{2+} close to the Ins(1,4,5)P_3-sensitive Ca^{2+} channel (Rizzuto et al., 1993). A second putative physiological role of Ca^{2+} cycling across Ca^{2+} stores might be the resulting sensitivity of $[Ca^{2+}]_i$ to modulation of the Ca^{2+}-ATPase activity. Thus it is conceivable that Ca^{2+} release in response to Ca^{2+}-ATPase inhibition is not a purely pharmacological phenomenon, but represents a mechanism that is used by certain agonists. Although such a mechanism has not been convincingly documented so far, the possibility deserves attention. For example, it has been suggested that arachidonic acid might induce Ca^{2+} release from intracellular stores through Ca^{2+}-ATPase inhibition (Chow and Jondal, 1990; Chan and Turk, 1987). In granulocytes, activation of the Fc-receptor-induced Ca^{2+} mobilization appears to be independent of Ins(1,4,5)P_3 generation, but may be blocked by low concentrations of a phospholipase A_2 inhibitor (Rosales and Brown, 1992). Thus $[Ca^{2+}]_i$ increase due to Ca^{2+}-ATPase inhibition might indeed be of physiological importance.

Note added in proof (received 6 June 1994)

A recent publication Smith and Gallacher, 1994 came to similar conclusions, using a different cell type and a different experimental approach. (Received 6 June 1994.)

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REFERENCES

Berridge, M. J. (1993) Nature (London) **361**, 315–325
Brune, B. and Ullrich, V. (1991) FEBS Lett. **284**, 1–4
Chan, K.-M. and Turk, J. (1987) Biochim. Biophys. Acta **928**, 186–193
Chow, S. C. and Jondal, M. (1990) J. Biol. Chem. **265**, 902–907
Demaurex, N., Lew, D. P. and Krause, K. H. (1992) J. Biol. Chem. **267**, 2318–2324
Demaurex, N., Grinstein, S., Jaconi, M. E. E., Schlegel, W., Lew, D. P. and Krause, K. H. (1993) J. Physiol. (London) **466**, 329–344
Demaurex, N., Monod, A., Lew, D. P. and Krause, K. H. (1994) Biochem. J. **297**, 595–601
Ghosh, T. K., Eis, P. S., Mullaney, J. M., Ebert, C. L. and Gill, D. L. (1988) J. Biol. Chem. **263**, 11075–11079
Hendey, B., Klee, C. B. and Maxfield, F. R. (1992) Science **258**, 296–299
Jaconi, M. E. E., Lew, D. P., Monod, A. and Krause, K. H. (1993) J. Biol. Chem. **268**, 26075–26078
Kass, G. E., Duddy, S. K., Moore, G. A. and Orrenius, S. (1989) J. Biol. Chem. **264**, 15192–15198
Krause, K. H. (1991) FEBS Lett. **285**, 225–229
Loomis-Husselbee, J. W. and Dawson, A. P. (1993) Biochem. J. **289**, 861–866
Mason, M. J., Garcia-Rodrigez, C. and Grinstein, S. (1991) J. Biol. Chem. **266**, 20856–20862
Missiaen, L., Parys, J. B., De Smedt, H. and Casteels, R. (1993) Biochem. Biophys. Res. Commun. **193**, 6–12
Newburger, P. E., Chovaniec, M. E., Greenberger, J. S. and Cohen, H. J. (1979) J. Cell Biol. **82**, 315–322
Peterson, G. L. (1977) Anal. Biochem. **83**, 346–356
Pittet, D., Schlegel, W., Lew, D. P., Monod, A. and Mayr, G. W. (1989) J. Biol. Chem. **264**, 18489–18493
Pittet, D., Di Virgilio, F., Pozzan, T., Monod, A. and Lew, D. P. (1990) J. Biol. Chem. **265**, 14256–14263
Pittet, D., Krause, K. H. and Lew, D. P. (1992) Adv. Second Messenger Phosphoprotein Res. **26**, 369–398
Prentki, M., Wollheim, C. B. and Lew, D. P. (1984) J. Biol. Chem. **259**, 13777–13782
Rasmussen, H., Barrett, P., Zawalich, W., Isales, C., Stein, P., Smallwood, J., McCarthy, R. and Bollag, W. (1989) Ann. N. Y. Acad. Sci. **568**, 73–80
Rechsteiner, M. (1992) Methods Enzymol. **149**, 42–48
Rizzuto, R., Brini, M., Murgia, M. and Pozzan, T. (1993) Science **262**, 744–747
Rosales, C. and Brown, E. J. (1992) J. Biol. Chem. **267**, 5265–5271
Smith, P. M. and Gallacher, D. V. (1994) Biochem. J. **299**, 37–40
Smith, C. D., Wen, D., Mooberry, S. L. and Chang, K.-J. (1992) Biochem. J. **281**, 803–808
Thastrup, O., Cullen, P. J., Drobak, B. K., Hanley, M. R. and Dawson, A. P. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 2466–2470
Van Delden, C., Favre, C., Spat, A., Cerny, E., Krause, K. H. and Lew, D. P. (1992) Biochem. J. **281**, 651–656

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